

Isolation and Identification of *Bifidobacteriaceae* from Human Saliva[▽]

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Received 20 April 2008/Accepted 11 August 2008

***Bifidobacteriaceae* were isolated from saliva and infected dentine by using a mupirocin-based selective medium. Of the saliva samples, 94% harbored bifids. The mean concentration (\pm the standard error) was 4.46 (± 0.12) \log_{10} (CFU per ml + 1), and the predominant isolates were *Bifidobacterium dentium*, *B. longum*, *Scardovia inopinata*, *Parascardovia denticolens*, and *Alloscardovia omnicolens*.**

The *Bifidobacteriaceae* constitute a significant proportion of the microbiota in the human gut (12), but the bifid populations of the oral cavity have been much less well studied. Little is known of their intraoral distribution or of the environmental factors, including dietary components, which may influence their numbers or the proportions of individual species isolated from the mouth. The cultivable commensal oral bifids are *Bifidobacterium dentium*, *Scardovia inopinata*, and *Parascardovia denticolens* (9, 20, 31), and *Scardovia* genomospecies strain C1 and *Scardovia* sp. strain T01-04 have also been described previously (16, 25). Oral bifids have been at best sporadically isolated, but when isolated or identified in 16S rRNA cloning and sequencing studies, they are usually found in carious dental tissues (1, 4, 7, 8, 11, 15, 17, 22–25, 33) but are not detected in healthy mouth tissues or in root caries lesion specimens (2, 26). There is inconsistency in the results of these previous studies. The aim of this study was to use conventional cultural methods to determine the prevalences and identities of oral bifids and, consequently, to investigate the genotypic diversity of *B. dentium* and *B. longum*.

We collected 5-min paraffin wax-stimulated saliva samples from 192 healthy dentate adult volunteers and infected dentine samples from the root caries lesions of 9 different subjects. The saliva samples were diluted in fastidious anaerobe broth (Lab M Ltd.). For the isolation of bifids, aliquots were spread onto a modified version of modified Trypticase-phytone-yeast extract (MTPY) medium (27), MMTPY, consisting of, per liter of deionized water, proteose peptone (Oxoid), 7 g; soya peptone (Oxoid), 5 g; yeast extract (Oxoid), 5 g; glucose, 15 g; raffinose, 5 g; cysteine-HCl, 0.5 g; K_2HPO_4 , 2 g; $MgCl_2 \cdot 6H_2O$, 0.5 g; $ZnSO_4 \cdot 7H_2O$, 0.25 g; $CaCl_2$, 0.15 g; $FeCl_3$, 0.001 g; agar, 15 g; and Tween 80, 1 ml. The medium was autoclaved at 15 lb/in² for 15 min and cooled, and 1 ml of glacial acetic acid and 1 ml of a 5-mg/ml mupirocin–50% ethanol solution were added. The dentine samples were diluted in Chalgren-Wilkins medium (Oxoid), and bifids were isolated using MMTPY.

Compared to MTPY medium, the MMTPY had a reduced concentration of mupirocin, from 100 to 5 μ g/ml, and modifications to the peptone source and raffinose to promote the isolation of non-glucose-fermenting species (5, 32). These modifications did not significantly affect the utility of the medium, as the growth of nonbifid organisms was inhibited and the counts of viable cells of type and reference strains were not significantly different from those on MTPY medium (data not shown). The numbers of mutans streptococci, lactobacilli, and yeasts (primarily *Candida* spp.) in the saliva samples were determined (39); the detection limit was 10 CFU per ml. The total number of presumptive bifid colonies on the MMTPY was established, a sample of each presumptive bifid colony type from 129 subjects was subcultured, and partial 16S rRNA gene sequences were obtained using the primers Bif164mod (5'-GGTGGTAATRCCSRATG-3') and Bif662mod (5'-CCACCGTTACACCGRGAA-3'), modified from those described previously (34), or the universal primers (21) 63F and 1387F. Sequences were determined with the ABI Prism BigDye Terminator version 3.0 cycle sequencing kit (Applied Biosystems) and analyzed by using information from the Ribosomal Database Project (<http://rdp.cme.msu.edu/>) or from BLAST searching. Bifids were isolated from 94% of participants, and the concentrations of bifids in saliva samples were not significantly different from those of mutans streptococci but were significantly greater than those of the other taxa (Table 1); the relative proportions of bifids and lactobacilli mirrored those in human adult feces (10). The improved detection of bifids was a consequence of the use of a selective medium and the observation that the 27F primer used in most 16S rRNA sequencing studies has three mismatches with the 27F sequence of all *Bifidobacteriaceae* (14), resulting in significantly reduced detection of this taxon. Approximately 50% of subjects had salivary bifid and mutans streptococcus counts of $>10^5$ (Fig. 1). The concentrations of all taxa in saliva samples correlated negatively with the salivary flow rate, and those of all taxa correlated significantly with one another (Table 1), suggesting that similar intraoral factors, including the salivary flow rate, may influence the concentrations of these organisms in saliva.

Of 354 presumptive bifid colonies identified, 341 (96.3%) were *Bifidobacteriaceae*. *B. dentium* was isolated from 106 subjects (82.2%). *S. inopinata* and *P. denticolens* were isolated

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[▽] Published ahead of print on 22 August 2008.

TABLE 1. Concentrations of bifids and caries-associated microorganisms in adult saliva samples and frequencies of isolation of these organisms^a

Type of organism	Frequency (%)	Concn (log ₁₀ [CFU/ml + 1]) in saliva samples (n = 192)			Spearman coefficient of correlation ^c with:			
		Mean ± SE	95% CI ^b	Median	Concn of mutans streptococci	Concn of lactobacilli	Concn of yeasts	Salivary flow rate
Bifids	94.2	4.46 ± 0.12	4.31–4.72	4.88	0.510	0.619	0.419	–0.250
Mutans streptococci	92.2	4.56 ± 0.13	4.26–4.73	5.08		0.586	0.534	–0.342
Lactobacilli	89.6	3.80 ± 0.14	3.54–4.06	4.25			0.547	–0.265
Yeasts	57.8	1.62 ± 0.11	1.41–1.82	1.61				–0.321

^a The concentrations of bifids in saliva samples were not significantly different from the levels of mutans streptococci but were significantly greater than the levels of lactobacilli ($P < 0.001$) and yeasts ($P < 0.001$) in the samples.

^b 95% CI, 95% confidence interval.

^c Coefficients of correlation between levels of bifids and levels of caries-associated microorganisms in saliva samples and between these levels and the salivary flow rate (measured in milliliters per min). All correlation coefficients correspond to P of <0.001 .

from six (4.7%) and four (3.2%) subjects, respectively, while *B. scardovii* and *Alloscardovia omnicolens* were each isolated from two subjects (1.6%). The identities of the predominant bifids were in accord with those in previous studies (9, 23, 30), but this is the first report of the isolation of *A. omnicolens* (19) and *B. scardovii* (18) from nonclinical specimens, suggesting that the mouth may be their normal habitat. *B. longum* was isolated from 14 subjects (10.8%), and from 8 subjects, only *B. longum* was isolated, with concentrations of up to $>10^7$ CFU per ml of saliva and a median of 4.4×10^5 CFU per ml. The salivary bifid concentrations in the samples from which *B. longum* was isolated were significantly greater than those in the other samples (mean ± standard error, 5.72 ± 0.22 versus 4.60 ± 0.12 log₁₀[CFU per ml + 1]; $P < 0.001$). *B. longum* isolates fermented (6) arabinose, glucose, and xylose, suggesting that they belonged to biovar longum (29).

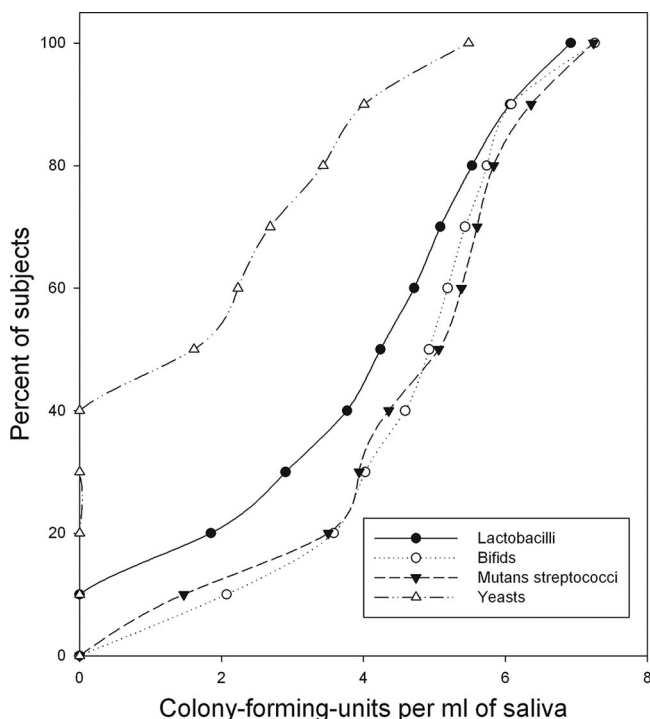


FIG. 1. Cumulative proportions of subjects ($n = 192$) colonized by bifids and caries-associated microorganisms.

To investigate the genotypic diversity of *B. dentium*, we examined 51 randomly selected independent isolates by repetitive extragenic palindromic PCR (REP-PCR) (3), and each isolate was distinct (similarity, $<90\%$) (Fig. 2A), similar to those of other oral commensal bacteria in previous studies (3, 13, 37). Individual root caries lesion samples harbored unique genotypes, and multiple genotypes were detected in each root caries lesion specimen (Fig. 2B). The isolation of *B. longum* from 10% of subjects indicates that there is a potential for *B. longum* derived from probiotic foods to establish in the mouth, although bifids consumed as probiotics do not persist in the normal fecal flora (28, 32). To ascertain whether the *B. longum* strains were the same and therefore likely to be from the same source, 12 independent isolates were genotyped using REP-PCR (3) and the BOX1AR primer, 5'-CTACGGCAAGGCG ACGCTGACG-3' (23). Neither genotyping method differentiated all isolates, but the combination of patterns demonstrated that all isolates were distinct (Fig. 3), suggesting independent origins for these strains. Thus, isolates Mu55-1 and Mu91-2 were similar by REP-PCR but were different by analysis with the BOX1AR primer. Repetitive-sequence PCRs have been used previously to identify individual species but not to assess intraspecies diversity (23, 38). In previous studies, three *B. dentium* isolates had apparently identical sequences as determined with the BOX1AR primer (23), while enterobacterial repetitive intergenic consensus-like primers gave similar band patterns for closely related species (35); here, REP-PCR primers (36) resulted in unique patterns for independent isolates, suggesting that REP-

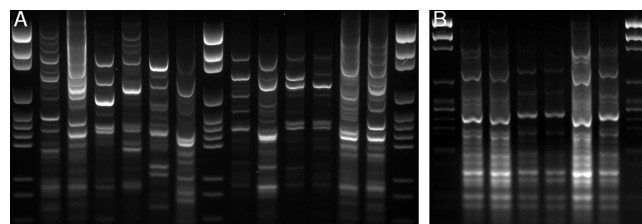


FIG. 2. (A) Comparison of representative independent *B. dentium* isolates examined by REP-PCR; (B) demonstration of different REP-PCR patterns among *B. dentium* isolates from the same root caries lesion specimen. Lanes 1, 8, and 14 (counting from the left) in panel A and lanes 1 and 8 (counting from the left) in panel B are molecular weight markers.

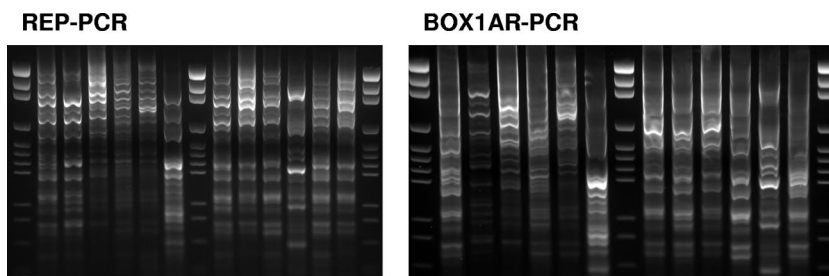


FIG. 3. Comparison of independent *B. longum* isolates examined by REP-PCR and BOX1AR PCR. Lanes 1, 7, and 14 (counting from the left) in both images are molecular weight markers. The isolates (13-4, Mu55-1, Mu57-1, Mu67-1, Mu79-1, Mu77-1, Mu91-2, Mu86-6, Mu92-1, Mu93-1, Mu100-6, and Mu38-2) are in the same order on each gel.

PCR may be useful for tracking isolates in epidemiological studies.

In conclusion, *Bifidobacteriaceae* were present at high levels in the saliva of adults and their numbers were significantly correlated with those of caries-associated organisms. Studies are now required to investigate the oral ecology of this taxon and to determine the utility of these organisms as markers of caries risk.

This project was funded in part by King's College London, The Biomedical Research Centre of Guy's and St. Thomas' Hospital Foundation Trust, and the Dunhill Medical Research Foundation. Glaxo-SmithKline generously supplied the mupirocin.

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